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(71) Applicant *(for all designated States except US)*: EUKARION, INC. [US/US]; 67 Crosby Street, Arlington, MA 02174 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : MALFROY-CAMINE, Bernard [FR/US]; 67 Crosby Street, Arlington, MA 02174 (US).

(74) Agents: DUNN, Tracy, J. et al.; Townsend and Townsend Khourie and Crew, One Market Plaza, 20th Fl., Steuart Street Tower, San Francisco, CA 94105 (US).

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(54) Title: TRANSVASCULAR AND INTRACELLULAR DELIVERY OF LIPIDIZED PROTEINS

(57) Abstract

The invention provides methods for targeting a protein, such as an antibody, to intracellular compartments in a eukaryotic cell, methods for enhancing organ uptake of proteins, pharmaceutical compositions of modified proteins for use in human therapy, and methods for manufacturing modified proteins. The modified proteins of the invention comprise an attached lipid protein, wherein one or more acyl groups are linked to the protein through a carbohydrate side-chain and various covalent linkage chemistries which are provided. Lipidized antibodies of the invention can be used for diagnostic and therapeutic uses.

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TRANSVASCULAR AND INTRACELLULAR DELIVERY OF LIPIDIZED PROTEINS

FIELD OF THE INVENTION

The invention provides methods for targeting a protein, such as an antibody, to intracellular compartments in a eukaryotic cell, methods for enhancing organ uptake of proteins, pharmaceutical compositions of modified proteins for use in human therapy, and methods for manufacturing modified proteins. The modified proteins of the invention comprise an attached lipid portion, wherein one or more acyl groups are linked to the protein through a carbohydrate side-chain and various covalent linkage chemistries which are provided.

BACKGROUND OF THE INVENTION

Many naturally-occurring or modified proteins have been proposed as diagnostic and/or therapeutic agents for use in humans and domestic animals. However, proteins are generally only poorly transported across vascular endothelial membranes, if at all, and usually cannot traverse cellular membranes to gain access to intracellular compartments. Thus, for example, antibodies can be raised against purified intracellular proteins, such as transcription factors, intracellular enzymes, and cytoarchitectural structural proteins, but such antibodies generally are not able to enter intact cells and bind to the intracellular antigen targets unless the cell membrane is disrupted.

The advent of monoclonal antibody technology in the mid 1970's heralded a new age of medicine. For the first time, researchers and clinicians had access to essentially unlimited quantities of uniform antibodies capable of binding to a predetermined antigenic site and having various immunological effector functions. These proteins, known as "monoclonal antibodies" were thought to hold great promise in, e.g., the removal of harmful cells, microbial pathogens, and viruses in vivo. Methods allowing the development of specific monoclonal antibodies having binding specificities directed

against almost any desired antigenic epitope, including antigens which are located in intracellular compartments in intact cells, promised a cornucopia of medicinal "magic bullets".

Unfortunately, the development of appropriate therapeutic products based on monoclonal antibodies, as well as polyclonal antisera, has been severely hampered by a number of drawbacks inherent in the chemical nature of naturally-occurring antibodies. First, antibodies are generally not able to efficiently gain access to intracellular locations, as immunoglobulins are not able to traverse the plasma membrane of cells, and are typically only internalized, if at all, as a consequence of inefficient endocytotic mechanisms. Second, antibodies do not generally cross vascular membranes (e.g., subendothelial basement membrane), hampering the efficient uptake of antibodies into organs and interstitial spaces. Therefore, therapies for many important diseases could be developed if there were an efficient method to get specific, biologically active immunoglobulin molecules across capillary barriers and into intracellular locations. For example, the life cycle of a retrovirus such as HIV involves intracellular replication wherein several viral-encoded polypeptides essential for production of infectious virions from an infected cell could potentially be inhibited or blocked if specific monoclonal antibodies reactive with the viral-encoded proteins could readily gain access to the intracellular locations where retroviral replication occurs.

Immunoliposomes have been produced as a potential targeted delivery system for delivering various molecules contained in the liposome to a targeted cell. Immunoliposomes employ immunoglobulins as targeting agents, wherein an acylated immunoglobulin is anchored in the lipid bilayer of the liposome to target the liposome to particular cell types that have external antigens that are bound by the acylated immunoglobulin(s) of the immunoliposomes (Connor and Huang (1985) J. Cell Biol. 101: 582; Huang, L. (1985) Biochemistry 24: 29; Babbitt et al. (1984) Biochemistry 23: 3920; Connor et

al. (1984) Proc. Natl. Acad. Sci. (U.S.A.) 81: 1715; Huang et al. (1983) J. Biol. Chem. 258: 14034; Shen et al. (1982) Biochim. Biphys. Acta 689: 31; Huang et al. (1982) Biochim. Biophys. Acta 716: 140; Huang et al. (1981) J. Immunol. Methods 46: 141; and Huang et al. (1980) J. Biol. Chem. 255: 8015). Immunoliposomes generally contain immunoglobulins which are attached to acyl substituents of a liposome bilayer through a crosslinking agent such as N-hydroxysuccimide and which thus become anchored in the liposome lipid bilayer. Hence, the crosslinked immunoglobulin is linked to the liposome and serves to target the liposomes to specific cell types bearing a predetermined external antigen by binding to the external cellular antigen. While such methods may serve to target liposomes to particular cell types, immunoliposomes suffer from several important drawbacks that have limited their application as drug-delivery vehicles, particularly for delivering proteins to intracellular locations.

Attempts have been made at modifying proteins so as to facilitate their transport across capillary barriers and into cells (EP 0 329 185), however, no completely satisfactory method has yet been reported in the art. Chemical modification of proteins, such as antibodies, by non-specific "cationization" to enhance transvascular and intracellular delivery of proteins, has been reported (U.S.S.N. 07/693,872). However, present methods for making cationized immunoglobulins lead to a significant loss of binding affinity (approximately about 90 percent) of a cationized immunoglobulin for binding to its predetermined epitope as compared to the comparable non-cationized immunoglobulin. Generally, cationization involves carbodiimide linkage of a diamine, such as putrescine or hexanediamine, to the carboxylates of aspartate and glutamate residues in the immunoglobulin polypeptide sequence. These chemical modifications of primary amino acids likely disrupt the secondary and tertiary structure of the immunoglobulin sufficiently to account for the loss in binding affinity. Also, present methods produce some degree of cationization in glutamate and aspartate residues located in

the variable domain of an immunoglobulin chain, which results in significant loss of binding affinity and/or specificity.

Chemical modification of small molecules has also been proposed as a method to augment transport of small bioactive compounds. Felgner (WO91/17242) discloses forming lipid complexes consisting of lipid vesicles and bioactive substances contained therein. Felgner et al. (WO91/16024) discloses cationic lipid compounds that are allegedly useful for enhancing transfer of small bioactive molecules in plants and animals. Liposomes and polycationic nucleic acids have been suggested as methods to deliver polynucleotides into cells. Liposomes often show a narrow spectrum of cell specificities, and when DNA is coated externally on to them, the DNA is often sensitive to cellular nucleases. Newer polycationic lipospermines compounds exhibit broad cell ranges (Behr et al., (1989) Proc. Natl. Acad. Sci. USA 86:6982) and DNA is coated by these compounds. In addition, a combination of neutral and cationic lipid has been indicated as a method for transfection of animal cells (Rose et al., (1991) BioTechniques 10:520).

Other approaches to enhancing delivery of drugs, particularly across the blood-brain barrier, utilize pharmacologic-based procedures involving drug latentiation or the conversion of hydrophilic drugs into lipid-soluble drugs. The majority of the latentiation approaches involve blocking the hydroxyl, carboxyl and primary amine groups on the drug to make it more lipid-soluble and therefore more easily transported across the blood-brain barrier. Pardridge and Schimmel, U.S. Patent 4,902,505, disclose chimeric peptides for enhancing transport by receptor-mediated transcytosis.

Thus, there exists a need in the art for methods of facilitating transport of specific proteins, such as antibodies, across capillary barriers and into cells, and for pharmaceutical compositions of such immunoglobulins for treating human and veterinary diseases which are amenable to treatment with intracellular proteins and targeting agents like monoclonal antibodies.

SUMMARY OF THE INVENTION

The prior art method of increasing antibody transport into cells by attaching a cationic substituent to the primary polypeptide sequence of an immunoglobulin by a relatively nonspecific linkage chemistry have been observed to produce detrimental alterations in the secondary, tertiary, and/or quaternary structure of the protein. These structural alterations apparently cause the loss of binding affinity observed in cationized antibodies. To overcome this, the present invention provides methods wherein lipid substituents are linked to a protein, such as an immunoglobulin, typically by covalent linkage to a carbohydrate side chain of the protein such that the lipid substituent does not substantially destroy the biological activity of the protein (e.g., antigen binding).

The invention provides methods for producing lipidized proteins, generally by lipidization of a carbohydrate moiety on a glycoprotein or glycopeptide. In general, the methods of the invention are used for attaching a lipid, such as a lipoamine, to a polypeptide, typically by covalent linkage of the lipid to a carbohydrate moiety on a protein, wherein the carbohydrate moiety generally is chemically oxidized and reacted with a lipoamine to form a lipidized protein. The resultant lipidized protein generally has advantageous pharmacokinetic characteristics, such as an increased capacity to cross vascular barriers and access parenchymal cells of various organs and an increased ability to access intracellular compartments. In one aspect of the invention, lipidization of proteins, such as antibodies directed against transcription factors (e.g., Fos, Jun, AP-1, OCT-1, NF-AT), enhances intranuclear localization of the lipidized protein(s).

The invention also provides methods for producing lipidized antibodies that are efficiently transported across capillary barriers and internalized into mammalian cells <u>in vivo</u>. The methods of the invention relate to methods for chemically attaching at least one lipid substituent (e.g.,

lipoamine) to a carbohydrate substituent on an immunoglobulin to produce a carbohydrate-linked lipidized immunoglobulin, wherein the lipidized immunoglobulin is capable of intracellular localization. In alternate embodiments of the invention, at least one lipid substituent (e.g., lipoamine) is covalently attached to a non-carbohydrate moiety on a protein or polypeptide (e.g., by formation of an amide linkage with a Asp or Glu residue side-chain carboxyl substituent or a thioester linkage with a Cys residue). Also, a fatty acid can be linked to an Arg or Lys residue by the side-chain amine substituents.

Similarly, lipid substitutents can be covalently attached to peptidomimetic compounds. Peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or "peptidomimetics" (Fauchere, J. (1986) Adv. Drug Res. 15: 29; Veber and Freidinger (1985) TINS p.392; and Evans et al. (1987) J. Med. Chem 30: 1229, which are incorporated herein by reference) and are usually developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (i.e., a polypeptide that has a biological or pharmacological activity), but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: $-CH_2NH-$, $-CH_2S-$, $-CH_2-CH_2-$, $-CH=CH-$ (cis and trans), $-COCH_2-$, $-CH(OH)CH_2-$, and $-CH_2SO-$, by methods known in the art and further described in the following references: Spatola, A.F. in "Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins," B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Spatola, A.F., Vega Data (March 1983), Vol. 1, Issue 3, "Peptide Backbone Modifications" (general review); Morley, J.S., Trends Pharm Sci (1980) pp. 463-468 (general review); Hudson, D. et al., Int J Pept Prot

Res (1979) 14:177-185 ($-CH_2NH-$, CH_2CH_2-); Spatola, A.F. et al., Life Sci (1986) 38:1243-1249 ($-CH_2-S$); Hann, M.M., J Chem Soc Perkin Trans I (1982) 307-314 (-CH-CH-, cis and trans); Almquist, R.G. et al., J Med Chem (1980) 23:1392-1398 ($-COCH_2-$); Jennings-White, C. et al., Tetrahedron Lett (1982) 23:2533 ($-COCH_2-$); Szelke, M. et al., European Appln. EP 45665 (1982) CA: 97:39405 (1982) ($-CH(OH)CH_2-$); Holladay, M.W. et al., Tetrahedron Lett (1983) 24:4401-4404 ($-C(OH)CH_2-$); and Hruby, V.J., Life Sci (1982) 31:189-199 ($-CH_2-S-$); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is $-CH_2NH-$. Such peptide mimetics may have significant advantages over polypeptide embodiments, including, for example: more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (e.g., a broad-spectrum of biological activities), reduced antigenicity, and others. Lipidization of peptidomimetics usually involves covalent attachment of one or more acyl chains, directly or through a spacer (e.g., an amide group), to non-interfering position(s) on the peptidomimetic that are predicted by quantitative structure-activity data and/or molecular modeling. Such non-interfering positions generally are positions that do not form direct contacts with the macromolecules(s) (e.g., receptors) to which the peptidomimetic binds to produce the therapeutic effect. Lipidization of peptidomimetics should not substantially interfere with the desired biological or pharmacological activity of the peptidomimetic.

The invention also relates to therapeutic and diagnostic compositions of lipidized proteins, such as lipidized antibodies, that can cross vascular membranes and enter the intracellular compartment, particularly lipidized antibodies that bind to intracellular immunotherapeutic targets, such as viral-encoded gene products that are essential components of a viral life cycle (e.g., HIV-1 Tat protein), to intracellular antigens that are biologically active (e.g., an oncogene protein such as c-fos, c-src, c-myc,

c-lck (p56), c-fyn (p59), and c-abl), and/or to transmembrane or extracellular antigens (e.g., polypeptide hormone receptors such as an IL-2 receptor, PDGF receptor, EGF receptor, NGF receptor, GH receptor, or TNF receptor). Other proteins which can be targeted by lipidized antibodies include, but are not limited to, the following: c-ras p21, c-her-2 protein, c-raf, any of the various G proteins and/or G-protein activating proteins (GAPs), transcription factors such as NF-AT, calcineurin, and cis-trans prolyl isomerases. The lipidized antibodies can be used to localize a diagnostic reagent, such as a radiocontrast agent or magnetic resonance imaging component, to a specific location in the body, such as a specific organ, tissue, body compartment, cell type, neoplasm, or other anatomical structure (e.g., a pathological lesion). The lipidized antibodies can also be used to localize linked therapeutic agents, such as chemotherapy drugs, radiosensitizing agents, radionuclides, antibiotics, and other agents, to specific locations in the body. Alternatively, the lipidized antibodies of the invention can be used therapeutically for neutralizing (i.e, binding to and thereby inactivating) an intracellular target antigen, such as HIV-1 Tat protein, a transmembrane or membrane-associated antigen target (e.g., γ-glutamyltranspeptidase, c-ras^H p21, rasGAP) or an extracellular antigen target (i.e., β-amyloid protein deposits in the brain of an Alzheimer's disease patient). Lipidized antibodies can traverse the blood-brain barrier and react with extracellular antigen targets that are generally inaccessible to immunoglobulins which circulate in the blood or lymphatic system. Lipidized antibodies can also react with intracellular portions on transmembrane proteins, such as cytoplasmic tails of viral envelope glycoproteins or protein kinase domains of protooncogene proteins (c-src, c-abl), and thus inhibit production of infectious enveloped virus or kinase activity, respectively.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows structural formulae representing

various lipoamines that can be used in the invention. The righthand column exemplifies branched-chain lipoamines and the lefthand column exemplifies straight-chain lipoamines.

Figure 2 is a schematic representation of (1) a glycosylated antibody comprising an immunoglobulin tetramer (two light chains associated with two heavy chains), and (2) a schematic representation of carbohydrate-linked lipidized immunoglobulins of the invention. For example but not limitation, branched-chain lipoamide substituents are shown attached to partially oxidized carbohydrate sidechains of an immunoglobulin tetramer. Such carbohydrate sidechains may be located in the C_H, V_H, C_L, and/or V_L regions.

Figure 3 shows the beneficial effect of a lipidized anti-Tat immunoglobulin on the <u>in vitro</u> survival of cells infected with HIV-1 as compared to the lack of effect of the native (i.e., non-lipidized) anti-Tat immunoglobulin.

Fig. 4 shows that the lipidized anti-Tat antibody significantly inhibited CAT activity (by approximately 75%), whereas native (unlipidized) anti-Tat antibody, lipidized anti-gp120 antibody, or rsCD4 were far less effective in inhibiting CAT activity in HLCD4-CAT cells.

DETAILED DESCRIPTION

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. For purposes of the present invention, the following terms are defined below.

As used herein, the twenty conventional amino acids and their abbreviations follow conventional usage (<u>Immunology - A Synthesis</u>, 2nd Edition, E.S. Golub and D.R. Gren, Eds., Sinauer Associates, Sunderland, Massachusetts (1991), which is incorporated herein by reference).

The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous (i.e., is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA".

The terms "substantial similarity" or "substantial identity" as used herein denotes a characteristic of a polypeptide sequence or nucleic acid sequence, wherein the polypeptide sequence has at least 50 percent sequence identity compared to a reference sequence, and the nucleic acid sequence has at least 70 percent sequence identity compared to a reference sequence. The percentage of sequence identity is calculated excluding small deletions or additions which total less than 25 percent of the reference sequence. The reference sequence may be a subset of a larger sequence, such as a constant region domain of a constant region immunoglobulin gene; however, the reference sequence is at least 18 nucleotides long in the case of polynucleotides, and at least 6 amino residues long in the case of a polypeptide.

The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring. A lipoprotein (e.g., a naturally-occuring isoprenylated or myristylated protein) that can be isolated from an organism that is found in nature and has not been engineered by man is a naturally-occurring lipoprotein.

"Glycosylation sites" refer to amino acid residues which are recognized by a eukaryotic cell as locations for the

attachment of sugar residues. The amino acids where carbohydrate, such as oligosaccharide, is attached are typically asparagine (N-linkage), serine (O-linkage), and threonine (O-linkage) residues. The specific site of attachment is typically signaled by a sequence of amino acids, referred to herein as a "glycosylation site sequence". The glycosylation site sequence for N-linked glycosylation is: -Asn-X-Ser- or -Asn-X-Thr-, where X may be any of the conventional amino acids, other than proline. The predominant glycosylation site sequence for O-linked glycosylation is: -(Thr or Ser)-X-X-Pro-, where X is any conventional amino acid. The recognition sequence for glycosaminoglycans (a specific type of sulfated sugar) is -Ser-Gly-X-Gly-, where X is any conventional amino acid. The terms "N-linked" and "O-linked" refer to the chemical group that serves as the attachment site between the sugar molecule and the amino acid residue. N-linked sugars are attached through an amino group; O-linked sugars are attached through a hydroxyl group. However, not all glycosylation site sequences in a protein are necessarily glycosylated; some proteins are secreted in both glycosylated and nonglycosylated forms, while others are fully glycosylated at one glycosylation site sequence but contain another glycosylation site sequence that is not glycosylated. Therefore, not all glycosylation site sequences that are present in a polypeptide are necessarily glycosylation sites where sugar residues are actually attached. The initial N-glycosylation during biosynthesis inserts the "core carbohydrate" or "core oligosaccharide" (Proteins, Structures and Molecular Principles, (1984) Creighton (ed.), W.H. Freeman and Company, New York, which is incorporated herein by reference).

As used herein, "glycosylating cell" is a cell capable of glycosylating proteins, particularly eukaryotic cells capable of adding an N-linked "core oligosaccharide" containing at least one mannose residue and/or capable of adding an O-linked sugar, to at least one glycosylation site sequence in at least one polypeptide expressed in said cell,

particularly a secreted protein. Thus, a glycosylating cell contains at least one enzymatic activity that catalyzes the attachment of a sugar residue to a glycosylating site sequence in a protein or polypeptide, and the cell actually glycosylates at least one expressed polypeptide. For example but not for limitation, mammalian cells are typically glycosylating cells. Other eukaryotic cells, such as insect cells and yeast, may be glycosylating cells.

As used herein, the term "antibody" refers to a protein consisting of one or more polypeptides substantially encoded by genes of the immunoglobulin superfamily (e.g., see <u>The Immunoglobulin Gene Superfamily</u>, A.F. Williams and A.N. Barclay, in <u>Immunoglobulin Genes</u>, T. Honjo, F.W. Alt, and T.H. Rabbitts, eds., (1989) Academic Press: San Diego, CA, pp.361-387, which is incorporated herein by reference). For example, but not for limitation, an antibody may comprise part or all of a heavy chain and part or all of a light chain, or may comprise only part or all of a heavy chain. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma (IgG_1, IgG_2, IgG_3, IgG_4), delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Full-length immunoglobulin "light chains" (about 25 Kd or 214 amino acids) are encoded by a variable region gene at the NH2-terminus (about 110 amino acids) and a kappa or lambda constant region gene at the COOH - terminus. Full-length immunoglobulin "heavy chains" (about 50 Kd or 446 amino acids), are similarly encoded by a variable region gene (about 116 amino acids) and one of the other aforementioned constant region genes, <u>e.g.</u>, gamma (encoding about 330 amino acids). Antibodies include, but are not limited to, the following: immunoglobulin fragments (e.g., Fab, $F(ab)_2$), Fv), single chain immunoglobulins, chimeric immunoglobulins, humanized antibodies, primatized antibodies, and various light chain-heavy chain combinations). Antibodies can be produced in glycosylating cells (e.g., human lymphocytes, hybridoma cells, yeast, etc.), in non-glycosylating cells (e.g., in *E. coli*), or synthesized by chemical methods or produced by <u>in vitro</u>

translation systems using a polynucleotide template to direct translation.

As used herein, a "lipidized antibody" is an antibody which has been modified by lipid derivatization (e.g., by covalent attachment of a lipoamine, such as glycyldioctadecylamide, dilauroylphosphatidylethanolamine, or dioctadecylamidoglycylspermidine) of one or more carbohydrate moieties attached to an immunoglobulin at a glycosylation site. Generally, the lipid substituent, such as a lipoamine, is covalently attached through a naturally-occurring carbohydrate moiety at a naturally-occurring glycosylation site. However, it is possible to produce immunoglobulins that have altered glycosylation site sequences (typically by site-directed mutagenesis of polynucleotides encoding immunoglobulin chains) and/or altered glycosylation patterns (e.g., by expression of immunoglobulin-encoding polynucleotides in glycosylating cells other than lymphocytes or in lymphocytes of other species). Lipid substituents can be attached to one or more naturally-occurring or non-naturally-occurring carbohydrate moiety on an immunoglobulin chain. When an antibody is produced by direct polypeptide synthesis or by biosynthesis in a non-glycosylating cell (e.g., a phage display library), it will generally be necessary to attach a carbohydrate substituent by chemical or enzymatic modification for subsequent lipidization (alternatively, the carbohydrate may be lipidized prior to attachment to the immunoglobulin).

As used herein, a "lipidized protein" refers to a protein (including multimeric proteins, glycoproteins, and polypeptides of various sizes) that has been modified by attachment of lipid (e.g., lipoamine), generally through a carbohydrate moiety. A lipidized protein is generated by derivatizing a protein such that the resultant lipidized protein is distinct from naturally-occurring lipid-linked proteins and lipoproteins. For proteins that are biologically active (e.g., enzymes, receptors, transcription factors), lipidization should not substantially destroy the biological

activity (e.g., at least about 15 percent of a native biological activity should be preserved in the lipidized protein). Lipidized peptidomimetics should retain at least about 25 to 95 percent of the pharmacologic activity of a corresponding non-lipidized peptidomimetic.

"Alkyl" refers to a fully saturated aliphatic group which may be cyclic, branched or straightchain. Alkyl groups include those exemplified by methyl, ethyl, cyclopropyl, cyclopropylmethyl, sec-butyl, heptyl, and dodecyl. All of the above can either be unsubstituted or substituted with one or more non-interfering substitutents, e.g., halogen; C_1-C_4 alkoxy; C_1-C_4 acyloxy; formyl; alkylenedioxy; benzyloxy; phenyl or benzyl, each optionally substituted with from 1 to 3 substituents selected from halogen, C_1-C_4 alkoxy or C_1-C_4 acyloxy. The term "non-interfering" characterizes the substituents as not adversely affecting any reactions to be performed in accordance with the process of this invention. If more than one alkyl group is present in a given molecule, each may be independently selected from "alkyl" unless otherwise stated.

"Alkylene" refers to a fully saturated divalent radical containing only carbon and hydrogen, and which may be a branched or straight chain radical. This term is further exemplified by radicals such as methylene, ethylene, n-propylene, t-butylene, i-pentylene, n-heptylene, and the like. All of the above can either be unsubstituted or substituted with one or more non-interfering sustituents, e.g., halogen; C_1-C_4 alkoxy; C_1-C_4 acyloxy; formyl; alkylenedioxy; benzyloxy; phenyl or benzyl, each optionally substituted with from 1 to 3 substituents selected from halogen, C_1-C_4 alkoxy or C_1-C_4 acyloxy. The term "non-interfering" characterizes the substituents as not adversely affecting any reactions to be performed in accordance with the process of this invention. If more than one alkylene group is present in a given molecule, each may be independently selected from "alkylene" unless otherwise stated.

"Aryl", denoted by Ar, includes monocyclic or

condensed carbocyclic aromatic groups having from 6 to 20 carbon atoms. Aryl groups include those exemplified by phenyl and naphthyl. These groups may be substituted with one or more non-interfering substituents, e.g., those selected from lower alkyl; lower alkenyl; lower alkynyl; lower alkoxy; lower alkylthio; lower alkylsulfinyl; lower alkylsulfonyl, dialkylamine; halogen; hydroxy; phenyl; phenyloxy; benzyl; benzoyl; and nitro. Each substituent may be optionally substituted with additional non-interfering substituents.

"Amino" refers to the group $-NH_2$.

"Alkylcarbonyl" refers to the group $-(CHR_1)-CO-$ wherein R_1 is further designated the α-position. R_1 may be hydrogen, alkyl, or an amino group. Preferably R_1 is an amino group.

Description of the Preferred Embodiments

In accordance with the present invention, novel methods for chemically modifying proteins, such as antibodies, to facilitate passage across capillary barriers and into cells are provided. In general, the methods include the covalent attachment of at least one non-interfering lipid substituent (e.g., glycyldioctadecylamide, glycyldiheptadecylamide, glycyldihexadecylamide, dilauroylphosphatidylethanolamine, and glycyldioctadecadienoylamide) to a reactive site in the protein molecule (e.g., a periodate-oxidized carbohydrate moiety). Various non-interfering lipid substituents may be attached to proteins to produce lipidized proteins, such as lipidized antibodies of the invention. For example but not for limitation, the following examples of lipids may be conjugated to a protein of interest to yield a lipidized protein: lipoamines, lipopolyamines, and fatty acids (e.g., stearic acid, oleic acid, and others). Generally, the lipid will be attached by a covalent linkage to a carbohydrate linked to the protein (e.g., a carbohydrate side chain of a glycoprotein). Naturally-occurring carbohydrate side-chains are preferably used for linkage to a lipoamine, although novel glycosylation sites may be engineered into a polypeptide by

genetic manipulation of an encoding polynucleotide, and expression of the encoding polynucleotide in a glycosylating cell to produce a glycosylated polypeptide.

Glycosylated proteins can be lipidized to enhance transvascular transport, organ uptake, and intracellular localization of the lipidized protein, including intranuclear localization. Generally, a glycosylated polypeptide, such as an antibody, is chemically oxidized with an oxidizing agent (e.g., periodate) to yield pendant carboxyl and/or aldehyde groups, and reacted with a lipoamine to form a covalent (amide or imide, respectively) bond linking the lipoamine to the protein. Typically, the oxidation of the carbohydrate side-chain is a partial oxidation producing at least one reactive carboxyl or aldehyde group, although generally chemical oxidation methods will produce some molecules that are partially oxidized and others that are either unoxidized or completely oxidized. However, in order to be lipidized by reaction with a lipoamine, the glycoprotein must be oxidized to produce at least one pendant aldehyde group that can react with a lipoamine, although it may be possible to produce lipidized proteins through linkage to pendant carboxyl groups as well. A pendant carboxyl or aldehyde group of an oxidized glycoprotein is a carboxyl or aldehyde group having a carbonyl carbon derived from an oxidized oligosaccharide and which is covalently attached to the protein, either directly or through a spacer (e.g., an unoxidized portion of a N- or O-linked carbohydrate side-chain). Preferably, N-linked and O-linked carbohydrate chains are incompletely oxidized to generate a multiplicity of reactive aldehyde and carboxyl groups at each glycosylation position for subsequent reaction with lipoamines. Most usually, glycoproteins having one or more complex N-linked oligosaccharides, such as those having a branched $(\text{mannose})_3(\beta\text{-N-acetylglucosamino})$ core, are partially oxidized by limited reaction with a suitable oxidant, generally periodate. Linked oligosaccharides containing N-acetylglucosamine (NAG), mannose, galactose, fucose (6-deoxygalactose), N-acetylneuraminic acid (sialic acid),

glucose, N-acetylmuramic acid, N-acetylgalactosamine, xylose, or combinations of these monosaccharide units can be oxidized and reacted with lipoamines to produce lipidized proteins, more specifically carbohydrate-linked lipidized proteins. Glycoproteins containing linked oligosaccharides with monosaccharide units other than those specifically listed above for exemplification, including non-naturally occurring monosaccharides, can also be oxidized and covalently linked to a lipoamine to form a lipidized protein.

Lipoamines are molecules having at least one acyl group and at least one free amine (i.e., a primary or secondary amine). It is believed that the invention can also be practiced with lipoamines that have tertiary amines which comprise at least one substituent that can be displaced by reaction with an oxidized carbohydrate. Examples of lipoamines having a primary amine are shown in Fig. 1. For example, the invention can produce lipidized proteins by reacting a glycoprotein with a straight-chain lipoamine of the formula:

$$NH_2\text{-}R\text{-}(CH_2)_n\text{-}CH_3$$

where R is: a disubstituted alkyl (alkylene), preferably methylene ($-CH_2-$); a 1,4-disubstituted cyclohexyl; a disubstituted aryl (arylene); preferably a 1,4-disubstituted phenyl (phenylene); an amido group of the formula $-(CHR_1)-CO-NH-$ wherein R_1 is hydrogen or an amino group; alkylcarbonyl, preferably α-amino substituted alkylcarbonyl; or a phosphate diester, preferably of the formula $-CH_2-O-PO_2-O-$. n is an integer which is typically 1 to 50, preferably about 5 to 30, more preferably about 10 to 25, and most usually about 15 to 20. In general, n is selected at the discretion of the practitioner according to the following guideline: when the molecule to be lipidized is large (i.e., a protein of more than about 10 kD) it is preferred than n is at least about 8 to 12 or more to increase the hydrophobicity of the resulting lipidized protein; when the molecule to be lipidized is small

(e.g., an oligopeptide) n can typically be in the range 2 to 18, but may be larger if additional hydrophobicity of the lipidized molecule is desired.

The invention can also be practiced with branched-chain lipoamines, which, for example, can include lipoamines of the formula:

$$\begin{array}{l} NH_2-R'-(CH_2)_n-CH_3 \\ \qquad\quad | \\ \qquad (CH_2)_m \\ \qquad\quad | \\ \qquad\ CH_3 \end{array}$$

where R' is: a trisubstituted alkyl, preferably $-CH_2-CH<$ or 1,2,4-trisubstituted cyclohexyl; a trisubstituted aryl, preferably 1,2,4-trisubstituted phenyl; an amido group of the formula $-(CHR_1)-CO-N<$ wherein R_1 is hydrogen or an amino group; an imino group of the formula $-CHR_2-NH-CH<$ wherein R_2 is hydrogen or an amino group or an imino group of the formula $-CH_2-N<$; or a phosphate diester, preferably of the formula $-CH_2-CH_2-O-PO_2-O-CH_2-CH(CO_2-)_2$. m and n are selected independently and are integers which are typically 1 to 50, preferably about 5 to 30, more preferably about 10 to 25, and most usually about 15 to 20. In general, n is selected at the discretion of the practitioner according to the following guideline: when the molecule to be lipidized is large (i.e., a protein of more than about 10 kD) it is preferred than m and/or n is at least about 8 to 12 or more to increase the hydrophobicity of the resulting lipidized protein; when the molecule to be lipidized is small (e.g., an oligopeptide) n can typically be in the range 2 to 18, but may be larger if additional hydrophobicity of the lipidized molecule is desired.

Essentially any glycoprotein can be lipidized according to the methods of the invention by reacting a lipoamine with an oxidized carbohydrate side-chain. Fig. 2 shows schematically a glycosylated antibody and a carbohydrate-linked lipidized antibody of the invention, respectively. Non-glycosylated proteins may be conjugated to a lipid by linkage through a suitable crosslinking agent (e.g., by carbodiimide linkage chemistry).

In accordance with the present invention, novel lipidized antibodies capable of specifically binding to predetermined intracellular epitopes with strong affinity are provided. These antibodies readily enter the intracellular compartment and have binding affinities of at least about $1 \times 10^6\ M^{-1}$, preferably $1 \times 10^7\ M^{-1}$ to $1 \times 10^8\ M^{-1}$, more preferably at least about $1 \times 10^9\ M^{-1}$ or stronger. The lipidized antibodies typically have a lipid substituent attached to a naturally-occurring carbohydrate side chain on a donor immunoglobulin chain, which composes an antibody specifically reactive with an intracellular, transmembrane, or extracellular epitope. Since carbohydrates are located on the Fc portion of immunoglobulins, chemical modification of the carbohydrate residues by lipidization would be unlikely to produce a substantial loss of affinity of the antibodies for their antigens (Rodwell et al. (1986) Proc. Natl. Acad. Sci. (U.S.A.) 83: 2632). The lipidized antibodies generally retain substantial affinity for their antigen, and the avidity can be readily measured by any of several antibody-antigen binding assays known in the art. The antibodies can be produced economically in large quantities and find use, for example, in the treatment of various human disorders by a variety of techniques.

One form of immunoglobulin constitutes the basic structural unit of an antibody. This form is a tetramer and consists of two identical pairs of immunoglobulin chains, each pair having one light and one heavy chain. In each pair, the light and heavy chain variable regions are together responsible for binding to an antigen, and the constant regions are responsible for the antibody effector functions. In addition to antibodies, immunoglobulins may exist in a variety of other forms including, for example, Fv, Fab, and $(Fab')_2$, as well as bifunctional hybrid antibodies, fusion proteins (e.g., bacteriophage display libraries), and other forms (e.g., Lanzavecchia et al., Eur. J. Immunol. 17, 105 (1987)) and in single chains (e.g., Huston et al., Proc. Natl. Acad. Sci. U.S.A., 85, 5879-5883 (1988) and Bird et al.,

Science, 242, 423-426 (1988)). (See, generally, Hood et al., "Immunology", Benjamin, N.Y., 2nd ed. (1984), and Hunkapiller and Hood, Nature, 323, 15-16 (1986)).

Antibodies can be produced in glycosylating cells (e.g., human lymphocytes, hybridoma cells, yeast, etc.), in non-glycosylating cells (e.g., in *E. coli*), or synthesized by chemical methods or produced by in vitro translation systems using a polynucleotide template to direct translation. One source of hybridoma cell lines and immunoglobulin-encoding polynucleotides is American Type Culture Collection, Rockville, MD. Methods for expression of heterologous proteins in recombinant hosts, chemical synthesis of polypeptides, and in vitro translation are well known in the art and are described further in Maniatis et al., Molecular Cloning: A Laboratory Manual (1989), 2nd Ed., Cold Spring Harbor, N.Y.; Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, CA; Merrifield, J. (1969) J. Am. Chem. Soc. 91: 501; Chaiken I.M. (1981) CRC Crit. Rev. Biochem. 11: 255; Kaiser et al.(1989) Science 243: 187; Merrifield, B. (1986) Science 232: 342; Kent, S.B.H. (1988) Ann. Rev. Biochem. 57: 957; and Offord, R.E. (1980) Semisynthetic Proteins, Wiley Publishing, which are incorporated herein by reference). Antibodies that are produced in non-glycosylating cells can be conjugated to a lipid by use of a bifunctional crosslinking agent or preferably post-translationally glycosylated in a glycosylation system such as purified canine pancreatic microsomes (Mueckler and Lodish (1986) Cell 44: 629 and Walter, P. (1983) Meth. Enzymol. 96: 84, which are incorporated herein by reference). Alternatively, polynucleotides that encode antibodies may be isolated from screened prokaryotic expression libraries, such as combinatorial antibody fragment display libraries, and subsequently expressed in glycosylating cells to produce glycosylated antibodies. According to these methods, glycosylated antibodies may be obtained, having naturally-occurring and/or non-naturally-occurring glycosylation

patterns. Such glycosylated antibodies can be lipidized according to the methods of the invention.

Glycosylation of immunoglobulins has been shown to have significant effects on their effector functions, structural stability, and rate of secretion from antibody-producing cells (Leatherbarrow et al., Mol. Immunol. 22: 407 (1985)). The carbohydrate groups responsible for these properties are generally attached to the constant (C) regions of the antibodies. For example, glycosylation of IgG at asparagine 297 in the C_H2 domain is required for full capacity of IgG to activate the classical pathway of complement-dependent cytolysis (Tao and Morrison, J. Immunol. 143: 2595 (1989)). Glycosylation of IgM at asparagine 402 in the C_H3 domain is necessary for proper assembly and cytolytic activity of the antibody (Muraoka and Shulman, J. Immunol. 142: 695 (1989)). Removal of glycosylation sites as positions 162 and 419 in the C_H1 and C_H3 domains of an IgA antibody lead to intracellular degradation and at least 90% inhibition of secretion (Taylor and Wall, Mol. Cell. Biol. 8: 4197 (1988)).

Glycosylation of immunoglobulins in the variable (V) region has also been observed. Sox and Hood, Proc. Natl. Acad. Sci. USA 66: 975 (1970), reported that about 20% of human antibodies are glycosylated in the V region. Glycosylation of the V domain is believed to arise from fortuitous occurrences of the N-linked glycosylation signal Asn-Xaa-Ser/Thr in the V region sequence and has not been recognized in the art as playing an important role in immunoglobulin function.

Therefore, it is generally preferred that lipidization is performed on antibodies having naturally-occurring glycosylation patterns. If glycosylation sites are engineered into an antibody, it is preferred that novel glycosylation site be introduced in a constant region or variable region framework region, which are less likely to adversely affect the antigen binding activity of the antibody. It is generally most preferred that novel glycosylation sites which are engineered into an antibody are placed in a constant

region.

Alternatively, polypeptide fragments comprising only a portion of a primary antibody structure and having a carbohydrate side chain that may be derivatized with a lipid substituent (e.g., lipoamine) can be produced, which fragments possess one or more immunoglobulin activities (e.g., antigen binding activity). These polypeptide fragments may be produced by proteolytic cleavage of intact antibodies by methods well known in the art, or by site-directed mutagenesis at the desired locations in expression vectors containing sequences encoding immunoglobulin proteins, such as after CH_1 to produce Fab fragments or after the hinge region to produce $(Fab')_2$ fragments. Single chain antibodies may be produced by joining V_L and V_H with a DNA linker (see, Huston et al., op. cit., and Bird et al., op. cit.). Also because like many genes, the immunoglobulin-related genes contain separate functional regions, each having one or more distinct biological activities, the genes may be fused to functional regions from other genes having novel properties. Nucleic acid sequences for producing immunoglobulins for the present invention are capable of ultimately expressing the desired antibodies and can be formed from a variety of different polynucleotides (genomic or cDNA, RNA, synthetic oligonucleotides, etc.) and components (e.g., V, J, D, and C regions), as well as by a variety of different techniques. Joining appropriate synthetic and genomic sequences is presently the most common method of production, but cDNA sequences may also be utilized (see, European Patent Publication No. 0239400 and L. Reichmann et al., Nature, 332, 323-327 (1988)).

Immunoglobulins and/or DNA sequences encoding immunoglobulin chains may be obtained, for example, by hybridoma clones which can be produced according to methods known in the art (Kohler and Milstein (1976) Eur. J. Immunol. 6: 511, incorporated herein by reference) or can be obtained from several sources ("ATCC Catalog of Cell Lines and Hybridomas", American Type Culture Collection, Rockville, MD,

which is incorporated herein by reference). DNA sequences encoding immunoglobulin chains can be obtained by conventional cloning methods known in the art and described in various publications, for example, Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., (1989), Cold Spring Harbor, N.Y. and Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, CA, which are incorporated herein by reference.

As stated previously, the DNA sequences will be expressed in hosts, typically glycosylating cells, after the sequences have been operably linked to (i.e., positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, e.g., tetracycline-resistance or G418-resistance, to permit detection of those cells transformed with the desired DNA sequences (see, e.g., U.S. Patent 4,704,362).

E. coli is one prokaryotic host useful particularly for cloning the DNA sequences of the present invention. Other microbial hosts suitable for use include bacilli, such as *Bacillus subtilis*, and other Enterobacteriaceae, such as *Salmonella*, *Serratia*, and various *Pseudomonas* species. In these prokaryotic hosts, one can also make expression vectors, which will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (trp) promoter system, a β-galactosidase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation. Proteins, such as antibodies, that are expressed in non-glycosylating cells can be post-translationally

glycosylated in a glycosylation system (Mueckler and Lodish, op.cit., which is incorporated herein by reference.

Other microbes, such as yeast, may also be used for expression. *Saccharomyces* is a preferred host glycosylating cell, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired.

In addition to microorganisms, mammalian tissue cell culture may also be used to express and produce the polypeptides of the present invention (see, Winnacker, "From Genes to Clones," VCH Publishers, N.Y., N.Y. (1987)). Eukaryotic cells are actually preferred, because a number of suitable host cell lines capable of secreting intact immunoglobulins have been developed in the art, and include the CHO cell lines, various COS cell lines, HeLa cells, preferably myeloma cell lines, etc, and transformed B-cells or hybridomas. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer (Queen et al., Immunol. Rev., 89, 49-68 (1986)), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40, Adenovirus, cytomegalovirus, Bovine Papilloma Virus, and the like.

The vectors containing the DNA segments of interest (e.g., the heavy and light chain encoding sequences and expression control sequences) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts. (See, generally, Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, (1982).)

Once expressed, the whole antibodies, their dimers,

individual light and heavy chains, or other immunoglobulin forms of the present invention, can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (see, generally, R. Scopes, "Protein Purification", Springer-Verlag, N.Y. (1982)). Substantially pure immunoglobulins of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically (including extracorporeally) or in developing and performing assay procedures, immunofluorescent stainings, and the like. (See, generally, Immunological Methods, Vols. I and II, Lefkovits and Pernis, eds., Academic Press, New York, N.Y. (1979 and 1981)).

In the methods of the invention, intact immunoglobulins or their binding fragments, such as Fab, are preferably used. Typically, lipidized antibodies will be of the human IgM or IgG isotypes, but other mammalian constant regions may be utilized as desired. Lipidized antibodies of the IgA, IgG, IgM, IgE, IgD classes may be produced. Preferably, the lipidized antibodies of the invention are human, murine, bovine, equine, porcine, or non-human primate antibodies, more preferably human or murine antibodies. The invention can be used to produce lipidized antibodies of various types, including but not limited to: chimeric antibodies, humanized antibodies, primatized antibodies, F_v fragments, toxin-antibody conjugates, isotope-antibody conjugates, and imaging agent-antibody conjugates. For *in vivo* imaging, lipidized antibodies are suitably labeled with a diagnostic label, administered to the patient, and their location determined at various times following administration. Various methods of labeling antibodies with diagnostic reporters (e.g., with Tc^{99}, other radioligands, radiocontrast agents or radio-opaque dye) are known in the art.

Proteins and oligopeptides (i.e., polypeptides comprising from 2 to about 50 amino acid residues attached in

peptidyl linkage) other than immunoglobulins can be lipidized according to the methods the invention. Naturally-occurring glycoproteins (e.g., γ-glutamyltranspeptidase, thrombomodulin, glucose transporter proteins) are preferred substrates for lipidization through carbohydrate linkage, although substantially any polypeptide can be lipidized by covalent attachment through a crosslinking agent (e.g., N-hydroxysuccimide) to a suitable amino acid side chain. In alternate embodiments of the invention, at least one lipid substituent (e.g., lipoamine) is covalently attached to a non-carbohydrate moiety on a protein or polypeptide (e.g., by formation of an amide linkage with a Asp or Glu residue side-chain carboxyl substituent or a thioester linkage with a Cys residue). Also, a fatty acid can be linked to an Arg or Lys residue by the side-chain amine substituents. Examples of non-glycosylated proteins which may be lipidized for enhancing transvascular and intracellular transport include, but are not limited to, the following proteins: c-fos, c-myc, c-src, NF-AT, and HMG CoA reductase. Naturally-occurring lipoproteins, such as native proteins which undergo physiological farnesylation, geranylgeranylation, and palmitylation are natural products and are not defined herein as "lipidized proteins".

The lipidized antibodies and pharmaceutical compositions thereof are particularly useful for parenteral administration, i.e., subcutaneously, intramuscularly or intravenously. The compositions for parenteral administration will commonly comprise a solution of the immunoglobulin or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering

agents, toxicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, human albumin, etc. The concentration of antibody in these formulations can vary widely, i.e., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for injection could be made up to contain 1 ml sterile buffered water, and 1-10 mgs of lipidized immunoglobulin. A typical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 150 mg of antibody. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980), which is incorporated herein by reference.

The lipidized proteins and antibodies of this invention can be frozen or lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immune globulins and art-known lyophilization and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilization and reconstitution can lead to varying degrees of activity loss (e.g., with conventional immune globulins, IgM antibodies tend to have greater activity loss than IgG antibodies) and that use levels may have to be adjusted to compensate.

The compositions containing the present lipidized proteins (e.g., antibodies) or a cocktail thereof can be administered for prophylactic and/or therapeutic treatments. In therapeutic application, compositions are administered to a patient in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate

to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the infection and the general state of the patient's own immune system, but generally range from about 1 to about 200 mg of antibody per dose, with dosages of from 5 to 25 mg being more commonly used. It must be kept in mind that the materials of this invention may generally be employed in serious disease states, that is life-threatening or potentially life-threatening situations.

In prophylactic applications, compositions containing the present immunoglobulins or a cocktail thereof are administered to a patient not already in a disease state to enhance the patient's resistance. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend upon the patient's state of health and general level of immunity, but generally range from 0.1 to 25 mg per dose.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of the lipidized proteins and/or lipidized antibody(ies) of this invention sufficient to effectively treat the patient.

For diagnostic purposes, the lipidized antibodies may either be labeled or unlabeled. Unlabeled antibodies can be used in combination with other labeled antibodies (second antibodies) that are reactive with the lipidized antibody, such as antibodies specific for human immunoglobulin constant regions. Alternatively, the lipidized antibodies can be directly labeled. A wide variety of labels may be employed, such as radionuclides, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, ligands (particularly haptens), radiocontrast agents, metal chelates, etc. Numerous types of diagnostic imaging applications are available and are well known to those skilled in the art. For example, but not for limitation, an antibody that binds to a tumor antigen (e.g., anti-CEA antibodies) may be lipidized and conjugated to a

radiocontrast agent or magnetic imaging material, injected into a human patient, and detected so as to localize the position of a tumor or metastatic lesion.

The lipidized immunoglobulins of the present invention can be used for diagnosis and therapy. By way of illustration and not limitation, they can be used to treat cancer, autoimmune diseases, or viral infections. For treatment of cancer, the antibodies will typically bind to an antigen expressed preferentially in certain cancer cells, such as c-myc gene product and others well known to those skilled in the art. Preferably, the lipidized antibody will bind to a mutant protein, such as a c-ras oncogene product having a pathogenic (e.g., neoplastic) sequence, such as a substitution at position 12, 13, 59, or 61 of the protein (e.g., a Ser at position 12 of $p21^{ras}$). For treatment of autoimmune disease, the antibodies will typically bind to an critical regulatory protein expressed primarily in activated T-cells, such as NF-AT, and many other intracellular proteins well known to those skilled in the art (e.g., see Fundamental Immunology, 2nd ed., W.E. Paul, ed., Raven Press: New York, NY, which is incorporated herein by reference). For treatment of viral infections, the antibodies will typically bind to a protein expressed in cells infected by a particular virus such as the various viral encoded polymerases and HIV-1 Tat, and many other viral proteins well known to those skilled in the art (e.g., see Virology, 2nd ed., B.N. Fields et al., eds., (1990), Raven Press: New York, NY, which is incorporated herein by reference).

Kits can also be supplied for use with the subject lipidized antibodies in the protection against or detection of a cellular activity or for the presence of a selected cell intracellular protein or the diagnosis of disease. Thus, the subject composition of the present invention may be provided, usually in a lyophilized form in a container, either alone or in conjunction with additional antibodies specific for the desired cell type. The lipidized antibodies, which may be conjugated to a label or toxin, or unconjugated, are included

in the kits with buffers, such as Tris, phosphate, carbonate, etc., stabilizers, biocides, inert proteins, e.g., serum albumin, or the like, and a set of instructions for use. Generally, these materials will be present in less than about 5% wt. based on the amount of active antibody, and usually present in total amount of at least about 0.001% wt. based again on the antibody concentration. Frequently, it will be desirable to include an inert extender or excipient to dilute the active ingredients, where the excipient may be present in from about 1 to 99% wt. of the total composition. Where a second antibody capable of binding to the lipidized antibody is employed in an assay, this will usually be present in a separate vial. The second antibody is typically conjugated to a label and formulated in an analogous manner with the antibody formulations described above, as well as typically also being lipidized itself.

The lipidized antibodies of the present invention are also suited for use in improved diagnostic methods and protein purification methods. For example, many intracellular proteins are unstable (e.g., short half-life, susceptible to proteolysis) or prone to aggregation (e.g., β-amyloid protein) making purification and/or diagnostic detection difficult. Lipidized antibodies are able to penetrate living cells and bind to specific intracellular target antigens; such antibody-antigen binding may stabilize the target antigen and block enzymes involved in degradation of the target antigen (e.g., proteases, ubiquitin-conjugating enzymes, glycosidases) facilitating detection and/or purification of the target antigen.

In one variation of the invention, a lipidized antibody which specifically binds to an intracellular target antigen is contacted with live cells comprising the intracellular target antigen under physiological conditions (e.g., cell culture conditions, somatic conditions) and incubated for a suitable binding period (e.g., from about 10 minutes to several hours). The lipidized antibody specifically binds to the target antigen forming an antigen-

antibody complex which is less susceptible to degradation and/or aggregation that is the target antigen itself. Typically, the cells are then fixed and permeabilized and the antigen-antibody complex, comprising the target antigen bound to the lipidized antibody, is detected, usually with a labeled secondary antibody that specifically binds the the lipidized antibody. Examples of preferred labels attached to the secondary antibody are: FITC, rhodamine, horseradish peroxidase conjugates, alkaline phosphatase conjugates, β-galctosidase conjugates, biotinyl moieties, radioisotopes, and the like. In some embodiments, the secondary antibody may be lipidized and the fixation and/or permeabilization steps may be omitted and replaced with substantial washing of the cell sample to remove non-specific staining. It may also be possible to use a lipidized, labeled primary antibody directly and omit the second antibody. Labelled protein A may also be substituted for a secondary antibody for the detection of the primary (lipidized) antibody.

Lipidized antibodies may also be used for intracellular therapy, such as for binding to a predetermined intracellular target antigen and modifying a biochemical property of the target antigen. For example, multi-subunit proteins, such as heteromultimeric proteins (e.g., transcription factors, G-proteins) or homodimeric proteins (e.g., polymerized tubulin) may possess a biochemical activity (e.g., GTPase activity) or other activity that requires intermolecular interaction(s) that may be blocked by a lipidized antibody that specifically binds to one or more subunits and prevents functional interaction of the subunits. For example, a lipidized anti-Fos antibody which binds to a portion of Fos (e.g., leucine zipper) required for binding to Jun to form a transcriptionally active AP-1 transcription factor (Fos/Jun heterodimer) may block formation of functional AP-1 and inhibit AP-1-mediated gene transcription. Also for example, a lipidized anti-ras antibody may bind to an epitope of ras which is required for its proper signal transduction function (e.g., a GTP/GDP-binding site, a portion of ras that

binds an accessory protein such as GAP, or the like), therebymodifying the activity of intracellular ras in living cells.

The following examples are offered by way of illustration, not by way of limitation.

EXPERIMENTAL EXAMPLES

EXAMPLE 1

Preparation of a Lipidized Bovine IgG

Glycyldioctadecylamide was obtained by linking a glycine residue to dioctadecylamine according to the method described by Behr et al. (1989) Proc. Natl. Acad. Sci. (U.S.A.) 86: 6982, which is incorporated herein by reference. Benzyloxycarbonyl-glycyl-p-nitrophenol at 1 equivalent and triethylamine at 1.1 equivalents in CH_2Cl_2 are reacted for 5 hours, followed by addition of H_2, 10% Pd/C in CH_2Cl_2/EtOH and reaction for 1 hour.

Two mg of bovine IgG (Sigma) were dissolved in 400 μl of 300 mM $NaHCO_3$ in a 1.5 ml Eppendorf vial. Fifty μl of a freshly prepared $NaIO_4$ solution (42 mg/ml in H_2O) were added and the vial was wrapped in aluminum foil and gently shaken for 90 min. at room temperature. The reaction medium was then loaded on a PD-10 column (Pharmacia) previously equilibrated with 10 mM Na_2CO_3 (fraction 1), and the column was eluted with 500 μl fractions. Fraction number 7 (between 3 ml and 3.5 ml) contained approximately 1.6 mg of bovine IgG as measured using the Bradford protein assay.

A solution of glycyldioctadecylamide in DMSO was prepared (5 mg of the lipid into 1 ml of DMSO, vigorously vortexed for several minutes). Under those conditions the lipid was not fully dissolved. Fifty μl of this solution were taken carefully (and did not contain any undissolved lipid) and were added to 350 μl of fraction 7 obtained as described above, in an Eppendorf vial. The vial was wrapped in aluminum foil, and the mixture was gently shaken for 20 h at room temperature.

One hundred μl of a solution of $NaBH_4$ (10 mg/ml in

H_2O) were then added. After one hour, 40 μl of a solution of ethanolamine (15 μl in 1 ml H2O) were added. After an additional 1 h, the reaction mixture was loaded on a PD-10 column previously equilibrated in 100 mM HEPES buffer, pH 8.5. The fraction containing the lipidized IgG (between 3 and 3.5 ml) was collected and stored on ice.

Labelling with ^{14}C-acetic anhydride

^{14}C-acetic anhydride (500 μCi, Amersham) in benzene ($10x10^6$ cpm/μl) was used. Two 5 μl aliquots were added to the 500 μl fraction containing the lipidized IgG at 10 min interval in an Eppendorf vial. The reaction was left on ice. A 500 μl solution of native IgG (800 μg in 100 mM HEPES, pH 8.5) was treated the same way.

After 30 min. the vials were warmed to 20 to 25°C, the ^{14}C-labelled IgGs were separated from free ^{14}C-acetate on a PD-10 column equilibrated with PBS. Radioactivity incorporated was of approximately $10x10^6$ cpm for 500 μg.

Organ uptake studies

Male swiss albinos mice (20g) were used. One hundred μl of ^{14}C-labeled lipidized IgG or ^{14}C-labeled control IgG in PBS (approximately 400,000 dpm each) were administered intravenously by tail vein injection. Mice were killed after 30 min or 3 h, their blood collected in EDTA-containing tubes, and their brain (minus cerebellum and brainstem), spleen, one kidney and one liver lobe were dissected. Organs were homogenized in 1 ml 10 mM Tris buffer, pH 7.4, and 500 μl aliquots were counted in a Beckman scintillation counter. Protein concentration in these homogenates was determined by the Bradford assay (Coomassie blue). The blood was centrifuged and 20 μl fractions of the plasma were counted. Table I shows the uptake of ^{14}C in the brain, liver, spleen and kidney, expressed as the ration of radioactivity in 1 μg protein of the organs divided by the radioactivity in 1 μl plasma (data expressed as μl/μg protein).

TABLE I

Organ	30 minutes		3 hours	
	Control	Lipidized	Control	Lipidized
Brain	.20 ± .03	.32 ± .08	.94 ± .05	1.75 ± .09
Kidney	1.46 ± .31	9.50 ± 2.19	1.17 ± .04	3.54 ± .22
Liver	1.26 ± .26	4.95 ± .82	.66 ± .04	7.59 ± .27
Spleen	1.08 ± .27	4.54 ± .76	1.13 ± .03	12.0 ± 2.15

Control groups of 4 and 6 mice at 30 min and 3 hr, respectively. Groups receiving the lipidized IgG were of 6 mice at both time points. Data are means ± s.e.m.

EXAMPLE 2

Inhibition of HIV-1 Cytotoxicity with Lipidized Anti-Tat Antibody

A monoclonal antibody which specifically binds the Tat protein of HIV-1 was lipidized according to the method described in Example 1, supra, involving periodate oxidation of carbohydrate on the antibody, followed by covalent attachment of glycyldioctadecylamide to yield a carbohydrate-lipidized antibody, which was eluted from the final PD-10 column with PBS.

Sup T1 cells were maintained in 24-well plates (100,000 cells per ml, in 2 ml of modified RPMI 1640 culture medium). Cells were kept in culture with either: (1) no additional treatment (two controls), (2) in the presence of added native anti-Tat antibody (15 μg/ml), or (3) in the presence of the lipidized anti-Tat antibody (11.7 μg/ml) during the first five days of the experiment. At the end of the first day, HIV-1 IIIB was added to one well of control cells and to the cultures containing native anti-Tat antibody-treated cells or lipidized anti-Tat antibody-treated cells. Viable cells were counted daily. The untreated, HIV-infected cells grew up to a density of approximately 500,000 cells per ml, and their number began to decrease after approximately eight days due to the cytotoxic effect of the virus.

Uninfected cells grew up to a density of approximately 1,000,000 cells per ml. Treatment of infected cells with the native anti-Tat antibody did not protect the cells from the cytotoxic effect of the virus. In contrast the lipidized anti-Tat antibody led to an almost complete protection of the cells from the cytopathic effects of the HIV-1 virus. This protection continued for at least about 5 days after the treatment with the lipidized antibody was interrupted. The results are presented in Fig. 3.

In another experiment, Sup T1 cells were maintained in culture as described in the previous example and kept in culture without any treatment and without any infection, infected with HIV-1 IIIB with no treatment, treated with the native anti-Tat antibody (1 μg/ml) and infected, or treated with the lipidized anti-Tat antibody (1 μg/ml) and infected. In the last three conditions, the virus was added at the end of the first day in culture. In the last two conditions, the native or lipidized antibody was present from day 1 until day 7.

The data presented in Table II show that, while the native anti-Tat antibody has little if any effect on viable cell number and reverse transcriptase activity, the lipidized antibody induced a significant protection of the cells in culture from the cytopathic effect of the virus and a significant decrease in reverse transcriptase activity. The latter suggests that the lipidized antibody could inhibit intracellular HIV-1 replication.

TABLE II

Effect of a Lipidized Anti-Tat Antibody on Viable Cell Number and Reverse Transcriptase Activity in Sup T1 Cells Infected with HIV-1

	Viable Cells ($x10^6$) Days in Culture						
Conditions	1	2	3	5	6	7	8
Untreated, Uninfected	1.15	1.28	1.52	1.63	1.69	1.76	1.72
Untreated, Infected	1.12	1.2	1.18	1.12	0.92	0.64	0.51
Treated with native anti-Tat Ab Infected	1.12	1.2	1.21	1.15	0.96	0.67	0.51
Treated with lipidized anti-Tat Ab Infected	1.15	1.26	1.3	1.36	1.17	0.99	0.75

	Reverse Transcriptase Activity ($cpm/10^9$ cells) Days in Culture						
Conditions	1	2	3	5	6	7	8
Untreated, Uninfected	2	2	1	2	2	1	1
Untreated, Infected	3	2	4	175	264	337	367
Treated with native anti-Tat Ab Infected	2	2	3	151	237	259	331
Treated with lipidized anti-Tab Ab Infected	3	2	2	57	135	179	184

HIV-1-infected SupT1 cells were treated daily with anti-Tat antibody in native or lipidized form or with rsCD4 (all proteins used at 1 μg/ml) starting from Day 1 before addition of HIV-1 virus containing supernatants until 10 days post infection. Cell numbers and reverse transcriptase activity (RT) in the culture medium were determined every day starting from Day 2 post-infection. By Day 10, the native anti-Tat still had no significant effect on either cell counts

or RT activity, whereas the lipidized anti-Tat antibody increased cell viability as compared to untreated, infected cells by approximately 70% and decreased RT activity by approximately the same extent. Cultures were continued for 3 days without further addition of antibodies. Effects of the lipidized anti-Tat persisted for the 3 days, indicating that the lipidized anti-Tat antibodies had accumulated in the cells in amounts high enough to provide sustained protection against viral infection/replication. The magnitude of the effects of the lipidized anti-Tat antibody on cell viability and RT activity were very similar to those observed with rsCD4 at the same dose. Increasing the concentration of the lipidized anti-Tat antibodies to 10 μg/ml did not induce further decrease in RT activity.

Example 3

Ability of Lipidized Anti-Tat to Inhibit the Transcriptional Activity of Tat on the HIV-1 LTR

A HeLa cell line stably transfected with a polynucleotide expressing CD4, the membrane receptor mediating HIV-1 infection, and also containing a reporter construct comprising an HIV-1 long terminal repeat (LTR) in operable linkage to and driving transcription of a linked reporter gene (chloramphenicol acetyltransferase; CAT). These cells (HLCD4-CAT) are susceptible to HIV-1 infection which produces functional Tat protein; the binding of newly synthesized Tat to the HIV-1 LTR leads to transcription of the linked CAT gene. Thus, the magnitude of CAT expression is approximately proportional to the extent of HIV-1 infection and the activity of Tat protein in the cells.

Cultured HeLa cells (3×10^5 cells/ml in DMEM with 10% fetal bovine serum) were exposed to the same concentrations (1 or 10 μg/ml) of various antibodies (in native or lipidized form) or recombinant soluble CD4 (rsCD4) for 1 hour and were extensively washed prior to addition of HIV-containing cell culture supernatants (100 μl). Twenty-four hours later the cells were harvested and CAT expression was measured by the method of Ho et al. (1984). Each experiment was run in quadruplicate and conducted four different time. Fig. 4 shows that the lipidized anti-Tat antibody significantly inhibited

CAT activity (by approximately 75%), whereas native (unlipidized) anti-Tat antibody, lipidized anti-gp120 antibody, or rsCD4 were far less effective in inhibiting CAT activity. These data indicate that lipidized anti-Tat was able to specifically bind its intracellular target, Tat, and inhibit the target's activity as a transcriptional activator of the LTR/reporter gene construct.

Moreover, the data showing passage of the lipidized anti-Tat antibody into HeLa cells indicates that the transport mechanism does not likely require endosome formation, since HeLa cells are reported to undergo little if any phagocytosis.

Example 4

Preparation of Lipidized Immunoglobulins Reactive with an Intracellular Protein

Glycyldioctadecylamide is obtained by linking a glycine residue to dioctadecylamine according to the method described by Behr et al. (1989) Proc. Natl. Acad. Sci. (U.S.A.) 86: 6982, which is incorporated herein by reference. Benzyloxycarbonyl-glycyl-p-nitrophenol at 1 equivalent and triethylamine at 1.1 equivalents in CH_2Cl_2 are reacted for 5 hours, followed by addition of H_2, 10% Pd/C in CH_2Cl_2/EtOH and reaction for 1 hour.

Anti-Human c-Myc Ig

Glycosylated murine immunoglobulins that bind specifically to human c-myc protein are prepared by separately culturing the hybridoma cell lines MYC CT9-B7.3 (ATCC CRL 1725), MYC CT 14-G4.3 (ATCC CRL 1727), and MYC 1-9E10.2 (ATCC CRL 1729) in RPMI 1640 with 10 percent fetal bovine serum under specified conditions (Evan et al. (1985) Mol. Cell. Biol. 5: 3610, incorporated herein by reference) and the monoclonal antibodies secreted are collected and purified by conventional methods known in the art.

About 2 mg of each purified monoclonal antibody are dissolved in 400 µl of 300 mM $NaHCO_3$ in a 1.5 ml Eppendorf vial. Fifty µl of a freshly prepared $NaIO_4$ solution (42 mg/ml in H2O) is added and the vial is wrapped in aluminum foil and

gently shaken for 90 min. at room temperature. The reaction medium is then loaded on a PD-10 column (Pharmacia) previously equilibrated with 10 mM Na_2CO_3 (fraction 1), and the column is eluted with 500 µl fractions. The fraction(s) containing at least approximately 500 µg of IgG as measured using the Bradford protein assay are collected.

A solution of glycyldioctadecylamide in DMSO is prepared (5 mg of the lipid into 1 ml of DMSO, vigorously vortexed for several minutes). Under those conditions the lipid is not fully dissolved. Fifty µl of this solution is taken carefully and added to 350 µl of the purified IgG fractions obtained as described above, in an Eppendorf vial. The vial is wrapped in aluminum foil, and the mixture is gently shaken for 20 h at room temperature.

One hundred µl of a solution of $NaBH_4$ (10 mg/ml in H_2O) is then added. After one hour, 40 µl of a solution of ethanolamine (15 µl in 1 ml H2O) is added. After an additional 1 h, the reaction mixture is loaded on a PD-10 column previously equilibrated in PBS. The fraction containing the lipidized murine anti-human-myc IgG (between 3 and 3.5 ml) is collected and stored on ice.

Anti-HMG CoA Reductase Ig

Glycosylated murine immunoglobulins that bind specifically to the intracellular enzyme HMG CoA reductase are prepared by separately culturing the hybridoma cell line A9 (ATCC CRL 1811) in DMEM with 4.5 g/l glucose, 5% horse serum and 2.5% fetal bovine serum as described (Goldstein et al. (1983) J. Biol. Chem. 258: 8450, incorporated herein by reference) and the monoclonal antibodies secreted are collected and purified by conventional methods known in the art.

About 2 mg of each purified monoclonal antibody are dissolved in 400 µl of 300 mM $NaHCO_3$ in a 1.5 ml Eppendorf vial. Fifty µl of a freshly prepared $NaIO_4$ solution (42 mg/ml in H2O) is added and the vial is wrapped in aluminum foil and gently shaken for 90 min. at room temperature. The reaction medium is then loaded on a PD-10 column (Pharmacia) previously equilibrated with 10 mM Na_2CO_3 (fraction 1), and the column is

eluted with 500 μl fractions. The fraction(s) containing at least approximately 500 μg of IgG as measured using the Bradford protein assay are collected.

A solution of glycyldioctadecylamide in DMSO is prepared (5 mg of the lipid into 1 ml of DMSO, vigorously vortexed for several minutes). Under those conditions the lipid is not fully dissolved. Fifty μl of this solution is taken carefully and added to 350 μl of the purified IgG fractions obtained as described above, in an Eppendorf vial. The vial is wrapped in aluminum foil, and the mixture is gently shaken for 20 h at room temperature.

One hundred μl of a solution of $NaBH_4$ (10 mg/ml in H_2O) is then added. After one hour, 40 μl of a solution of ethanolamine (15 μl in 1 ml H2O) is added. After an additional 1 h, the reaction mixture is loaded on a PD-10 column previously equilibrated in PBS. The fraction containing the lipidized anti-HMG CoA reductase IgG (between 3 and 3.5 ml) is collected and stored on ice.

Example 5

Preparation of Lipidized Immunoglobulins Reactive with a Transmembrane Protein

Glycyldioctadecylamide is obtained by linking a glycine residue to dioctadecylamine according to the method described by Behr et al. (1989) Proc. Natl. Acad. Sci. (U.S.A.) 86: 6982, which is incorporated herein by reference. Benzyloxycarbonyl-glycyl-p-nitrophenol at 1 equivalent and triethylamine at 1.1 equivalents in CH_2Cl_2 are reacted for 5 hours, followed by addition of H_2, 10% Pd/C in CH_2Cl_2/EtOH and reaction for 1 hour.

Anti-Ras Ig

Glycosylated murine immunoglobulins that bind specifically to ras oncogene protein are prepared by separately culturing the hybridoma cell line 142-24E5 (ATCC HB 8679; U.S. Pats. 5,015,571 and 5,030,565, incorporated herein by reference) in DMEM with 4.5 g/l glucose, 2mM L-glutamine, 1mM sodium pyruvate, non-essential amino acids, 1xBME vitamins, 0.1

mM hypoxanthine, 0.032mM thymidine, 0.05 mg/ml gentamicin, and 10% fetal bovine serum, and the hybridoma cell MX (ATCC HB 9158) in Iscove's DMEM with 1% L-glutamine and HT and 10 percent fetal bovine serum under specified conditions (U.S. Patent 4,820, 631, incorporated herein by reference) and the monoclonal antibodies secreted from the hybridoma lines are collected and purified by conventional methods known in the art.

About 2 mg of each purified monoclonal antibody are dissolved in 400 μl of 300 mM $NaHCO_3$ in a 1.5 ml Eppendorf vial. Fifty μl of a freshly prepared $NaIO_4$ solution (42 mg/ml in H2O) is added and the vial is wrapped in aluminum foil and gently shaken for 90 min. at room temperature. The reaction medium is then loaded on a PD-10 column (Pharmacia) previously equilibrated with 10 mM Na_2CO_3 (fraction 1), and the column is eluted with 500 μl fractions. The fraction(s) containing at least approximately 500 μg of IgG as measured using the Bradford protein assay are collected.

A solution of glycyldioctadecylamide in DMSO is prepared (5 mg of the lipid into 1 ml of DMSO, vigorously vortexed for several minutes). Under those conditions the lipid is not fully dissolved. Fifty μl of this solution is taken carefully and added to 350 μl of the purified IgG fractions obtained as described above, in an Eppendorf vial. The vial is wrapped in aluminum foil, and the mixture is gently shaken for 20 h at room temperature.

One hundred μl of a solution of $NaBH_4$ (10 mg/ml in H_2O) is then added. After one hour, 40 μl of a solution of ethanolamine (15 μl in 1 ml H2O) is added. After an additional 1 h, the reaction mixture is loaded on a PD-10 column previously equilibrated in PBS. The fraction containing the lipidized murine anti-ras IgG (between 3 and 3.5 ml) is collected and stored on ice.

Hybridoma cell lines referred to in the above examples may be obtained from American Type Culture Collection, Rockville, MD (ATCC Cell Lines and Hybridomas (1992) 7th Ed, which is incorporated herein by reference).

Example 6

Lipidization of a Transmembrane Enzyme

The enzyme gamma-glutamyltranspeptidase (GGT: EC 2.3.2.2) is a widely distributed enzyme that catalyzes the degradation of glutathione and other γ-glutamyl compounds by hydrolysis of the γ-glutamyl moiety or by its transfer to a suitable acceptor. GGT is a heterodimeric glycoprotein, which is synthesized as a precursor protein that is glycosylated and cleaved into the two subunits of the mature enzyme. GGT is anchored to the cell membrane through the N-terminal portion of its heavy subunit. The active site of the enzyme lies on the extracellular portion of the molecule, which is heavily glycosylated.

GGT is separately purified from rat kidney and a cultured human hepatoma cell line according to procedures described previously in the art (Barouki et al. (1984) J. Biol. Chem. 259: 7970; Curthoys and Hughey (1979) Enzyme 24: 383; Matsuda et al. (1983) J. Biochem. 93: 1427; Taniguchi et al. (1985) J. Natl. Cancer Inst. 75: 841; Tate and Meister (1985) Methods Enzymol. 113: 400; and Toya et al. (1983) Ann. N.Y. Acad. Sci. 417: 86, which are incorporated herein by reference).

About 1 mg of each of the purified rat and human GGT preparations are dissolved in 400 μl of 300 mM $NaHCO_3$ in a 1.5 ml Eppendorf vial. Fifty μl of a freshly prepared $NaIO_4$ solution (42 mg/ml in H2O) is added and the vial is wrapped in aluminum foil and gently shaken for 60 min. at room temperature. The reaction medium is then loaded on a PD-10 column (Pharmacia) previously equilibrated with 10 mM Na_2CO_3 (fraction 1), and the column is eluted with 500 μl fractions. The fraction(s) containing at least approximately 100 μg of GGT as measured using the Bradford protein assay are collected.

A solution of glycyldioctadecylamide in DMSO is prepared (5 mg of the lipid into 1 ml of DMSO, vigorously vortexed for several minutes). Under those conditions the lipid is not fully dissolved. Fifty μl of this solution is taken carefully and added to 350 μl of the purified GGT fractions obtained as described above, in an Eppendorf vial.

The vial is wrapped in aluminum foil, and the mixture is gently shaken for 20 h at room temperature.

One hundred μl of a solution of $NaBH_4$ (10 mg/ml in H_2O) is then added. After one hour, 40 μl of a solution of ethanolamine (15 μl in 1 ml H2O) is added. After an additional 1 h, the reaction mixture is loaded on a PD-10 column previously equilibrated in PBS. The fraction containing the lipidized human and rat GGT (between 3 and 3.5 ml) is collected and stored on ice.

The lipidized human and rat GGT fractions are assayed for γ-glutamyltranspeptidase activity by conventional assay procedures (Tate and Meister (1983) op.cit., incorporated herein by reference) and the specific activity of the lipidized human GGT and lipidized rat GGT is determined.

The lipidized human and rat GGT is radiolabeled by iodination with ^{125}I using chloramine T and approximately 50 μg of the radioiodinated lipidized GGT is administered to rats by intraperitoneal injection. After 24 hours, the rats are sacrificed and tissue samples removed for autoradiography to determine the pattern of localization of the lipidized GGT in the various organs.

Example 7

Lipidization of an Anti-Actin Antibody and Intracellular Immunostaining

In order to demonstrate that lipidized antibodies can localize intracellularly in living cells and bind intracellular targets, an anti-actin antibody was lipidized and evaluated for its ability to penetrate cultured Swiss 3T3 fibroblasts and bind to the cytoskeletal protein actin. Native anti-actin antibody (unlipidized) was used as a control.

Protein A-purified rabbit anti-actin polyclonal antibodies were lipidized according to the following procedure. A lipoamine, glycyldioctadecylamide, was covalently linked to the carbohydrate moieties of the anti-actin antibodies by periodate oxidation-sodium borohydride reduction. Antibodies were dissolved in 0.8 ml of 300 mM $NaHCO_3$ at a concentration of approximately 0.2 to 1.0 mg/ml. Fifty μl of a freshly prepared

aqueous solution of $NaIO_4$ (42 mg/ml) were added and the incubation vials were wrapped in aluminum foil and gently shaken for 90 minutes at room temperature. The reaction mixture was then purified on a PD-10 column (Pharmacia, Piscataway, NJ) equilibrated in and eluted with 10 mM Na_2CO_3. Fifty μl of a 10 mg/ml solution of glycyldioctadecylamide in benzene are added to the fraction containing the antibodies (e.g., as determined by A_{280} monitoring, Bradford assay) and the resulting reaction was incubated for 20 hours at room temperature with gentle shaking. One hundred μl of a freshly prepared aqueous solution of $NaBH_4$ (10 mg/ml) was then added and inubated at room temperature for one hour, followed by addition of 50 μl of ethanolamine solution (15 μl ethanolamine dissolved in 1 ml of H_2O). After an additional hour at room temperature, the resultant lipidized antibodies were purified by chromatography on a PD-10 column equilibrated with phosphate-buffered saline.

ELISA Assays

Lipidized anti-actin and lipidized anti-Tat (supra) were evaluated for their binding affinity for specific antigen relative to native (unlipidized) anti-actin or anti-Tat antibody by ELISA assay. Lipidization of either the anti-actin antibody or the anti-Tat antibody did not produce a measurable loss of affinity of the antibodies for their respective antigens as compared to their native (unlipidized) antibody.

Intracellular Immunostaining

To demonstrate that lipidized anti-actin antibodies are able to bind intracellular actin in live cells, lipidized anti-actin antibody or native anti-actin antibody were contacted with cultured Swiss 3T3 cells for 1 hour, followed by extensive washing to remove residual anti-actin antibodies. The cells were subsequently fixed and permeabilized and the anti-actin antibodies were detected with a fluorescent-labeled secondary antibody. While no specific staining could be detected in cells preincubated with the native (unlipidized) anti-actin antibody, specific actin staining (e.g., stained actin cables) was clearly evident in cells preincubated with the lipidized anti-actin antibodies. The staining pattern

observed with the lipidized anti-actin antibody applied prior to fixation was similar to that seen using native (unlipidized) anti-actin antibody incubated with the cells following fixation and permeabilization. These data demonstrate that the lipidized anti-actin antibodies were able to reach and bind intracellular actin, and that they could still be recognized and bound by the labeled secondary antibodies, indicating that they were functional and substantially intact.

Although the present invention has been described in some detail by way of illustration for purposes of clarity of understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the claims.

CLAIMS

1. A method for modifying the pharmacokinetic characteristics of a protein, comprising the steps of:

attaching a lipid substituent to the protein by a covalent linkage to produce a lipidized protein; and

recovering the lipidized protein.

2. A method according to Claim 1, wherein the lipid substituent is a lipoamine.

3. A method according to Claim 2, wherein the step of attaching further comprises the steps of

oxidizing a carbohydrate on a glycosylated polypeptide to produce an oxidized glycoprotein; and

reacting the oxidized glycoprotein with a lipoamine under suitable reaction conditions to form a lipidized protein.

4. A method according to Claim 2, wherein the lipoamine is a straight-chain lipoamine according to the formula:

$$NH_2\text{-}R\text{-}(CH_2)_n\text{-}CH_3$$

where R is selected from the group consisting of: disubstituted alkyl (alkylene); 1,4-disubstituted cyclohexyl; disubstituted aryl (arylene); amido group of the formula $-(CHR_1)\text{-}CO\text{-}NH\text{-}$ wherein R_1 is hydrogen or an amino group; alkylcarbonyl; and phosphate diester; n is 1-50.

5. A method according to Claim 2, wherein the lipoamine is a branched-chain lipoamine according to the formula:

$$\begin{array}{l} NH_2\text{-}R'\text{-}(CH_2)_n\text{-}CH_3 \\ \qquad\;\; | \\ \qquad (CH_2)_m \\ \qquad\;\; | \\ \qquad\; CH_3 \end{array}$$

where R' is: a trisubstituted alkyl; a trisubstituted aryl; an amido group of the formula $-(CHR_1)\text{-}CO\text{-}N<$ wherein R_1 is

hydrogen or an amino group; an imino group of the formula $-CHR_2-NH-CH<$ wherein R_2 is hydrogen or an amino group or an imino group of the formula $-CH_2-N<$; or a phosphate diester; m is 1-50; n is 1-50; and m and n are selected independently.

6. A method according to claim 5, wherein the branched-chain lipoamine is glycyldioctadecylamide.

7. A method according to Claim 1, wherein the protein is a naturally-occurring glycoprotein.

8. A method according to Claim 1, wherein the protein is encoded by an immunoglobulin superfamily gene.

9. A method according to Claim 8, wherein the immunoglobulin superfamily gene encodes a μ or γ heavy chain.

10. A method according to Claim 7, wherein the naturally-occurring glycoprotein is an antibody.

11. A method according to Claim 10, wherein the antibody is a monoclonal antibody.

12. A method according to Claim 1, wherein the lipidized protein comprises at least one lipoamine residue linked to a carbohydrate side chain.

13. A method for targeting an intracellular protein for binding with an antibody in a cell, comprising contacting the cell with a lipidized antibody which binds specifically with the intracellular protein.

14. A method according to Claim 13, wherein the lipidized antibody comprises at least one lipoamine residue linked to a carbohydrate side chain of an immunoglobulin.

15. A method according to Claim 14, wherein the lipoamine is

glycyldioctadecylamide.

16. A method according to Claim 13, wherein the lipidized antibody is administered to a nonhuman animal *in vivo*.

17. A method according to Claim 16, wherein the lipidized antibody is taken up into an organ to a greater extent than is a comparable naturally-occurring antibody having the same amino acid sequence(s) and the same glycosylation pattern.

18. A composition for therapy or prophylaxis of a disease, comprising a therapeutically effective dosage of a lipidized protein.

19. A composition according to Claim 18, wherein the lipidized protein is an antibody.

20. A composition according to Claim 19, wherein the antibody binds to an intracellular protein.

21. A composition according to Claim 20, wherein the intracellular protein is a viral-encoded protein.

22. A composition according to Claim 21, wherein the viral-encoded protein is a Tat protein encoded by HIV-1.

23. A composition for prophylaxis, comprising a prophylactically effective dosage of a lipidized antibody.

24. A composition comprising a lipidized antibody linked to a diagnostic reporter.

25. A composition according to Claim 18, containing a carbohydrate-linked lipidized protein.

26. A composition according to Claim 25, wherein the carbohydrate-linked lipidized protein is a carbohydrate-linked lipidized immunoglobulin.

27. A pharmaceutically acceptable composition comprising a carbohydrate-linked lipidized immunoglobulin, and an excipient.

28. A method for diagnosing a pathological condition, comprising the steps of:

administering a lipidized antibody comprising a diagnostic reporter to a patient; and

detecting a location at which the diagnostic reporter is preferentially localized.

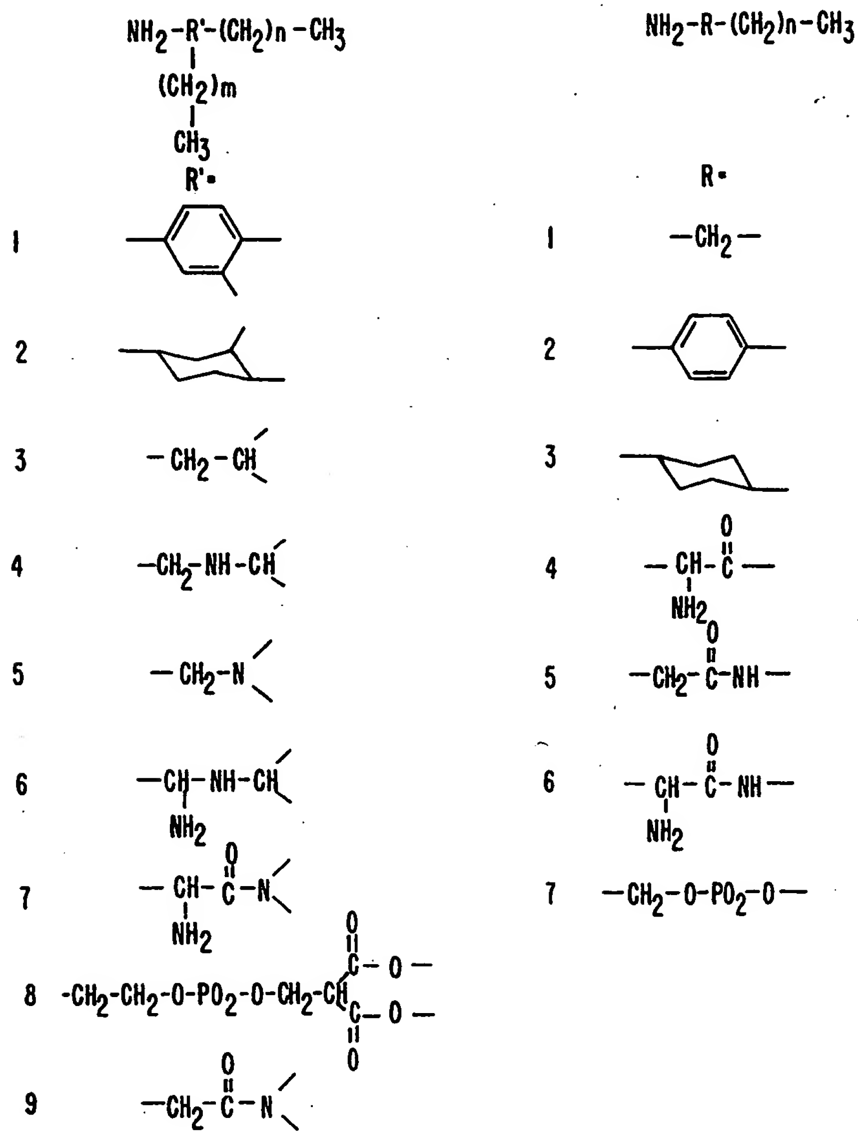
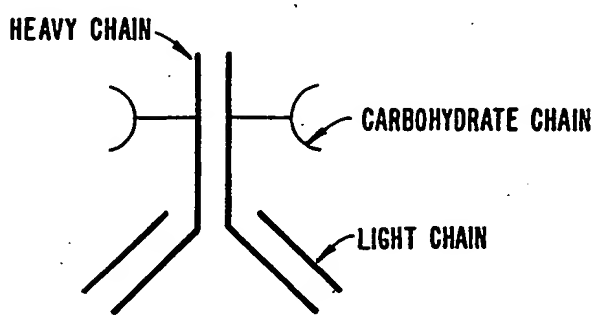
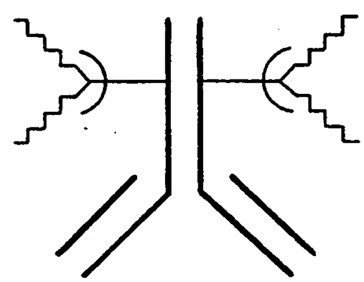


FIG. 1.



GLYCOSYLATED ANTIBODY



CARBOHYDRATE-LINKED LIPIDIZED ANTIBODY

FIG. 2.

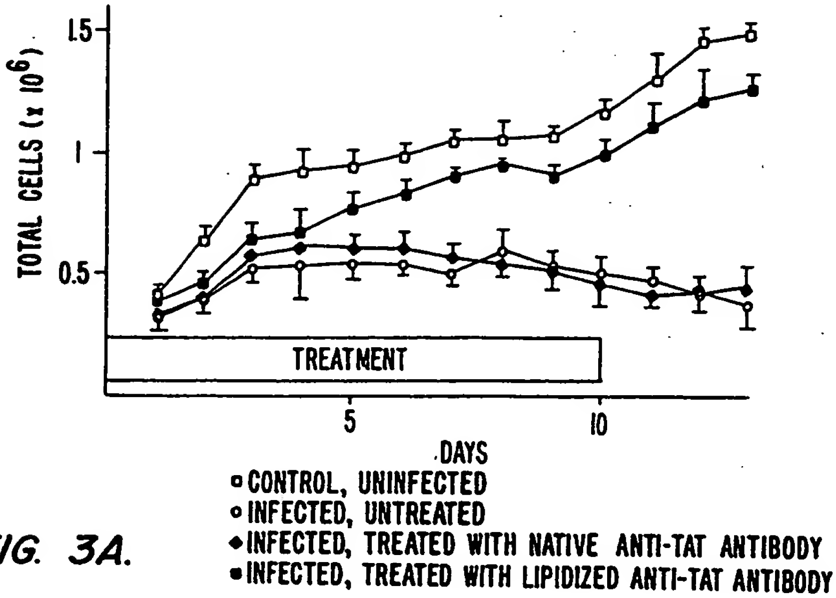

1.5
1
0.5
TOTAL CELLS (x 10^6)
TREATMENT
5
10
DAYS
CONTROL, UNINFECTED
INFECTED, UNTREATED
INFECTED, TREATED WITH NATIVE ANTI-TAT ANTIBODY
INFECTED, TREATED WITH LIPIDIZED ANTI-TAT ANTIBODY

FIG. 3A.

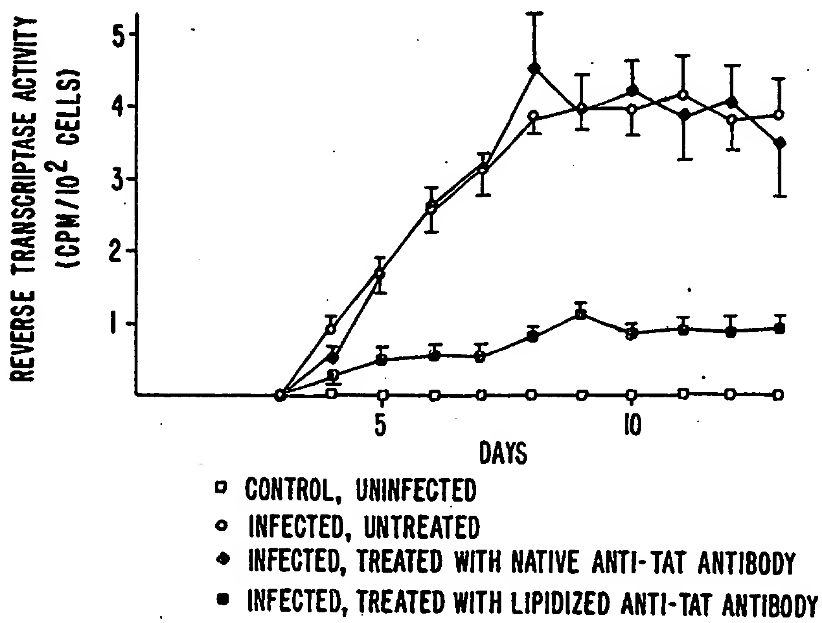
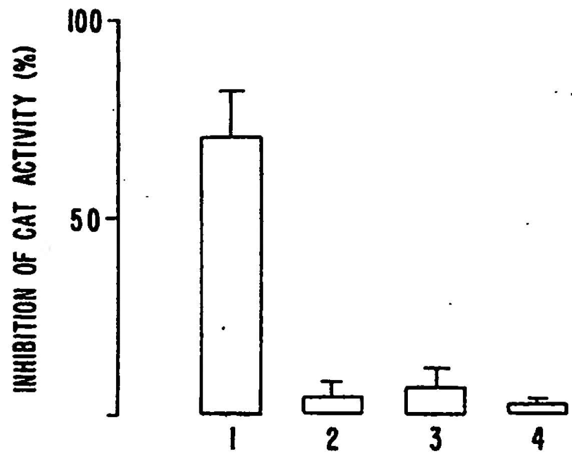

5
4
3
2
1
REVERSE TRANSCRIPTASE ACTIVITY
(CPM/10^2 CELLS)
5
10
DAYS
CONTROL, UNINFECTED
INFECTED, UNTREATED
INFECTED, TREATED WITH NATIVE ANTI-TAT ANTIBODY
INFECTED, TREATED WITH LIPIDIZED ANTI-TAT ANTIBODY

FIG. 3B.



1: LIPIDIZED ANTI-TAT ANTIBODY
2: NATIVE ANTI-TAT ANTIBODY
3: LIPIDIZED ANTI-GP120 ANTIBODY
4: SOLUBLE CD4

FIG. 4.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06599

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/00, 39/395, 35/14, 39/44; C07K 3/00, 17/00
US CL :424/85.8; 530/388.3, 391.1, 391.3, 391.5, 391.9, 359

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.8; 530/388.3, 391.1, 391.3, 391.5, 391.9, 359

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline, Biosis, Chem Ab, Embase, Derwent WPI, Medtext, search terms: lipid, antibody, intracellular, delivery, target, conjugate, author name

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Science USA, Volume 83, issued April 1986, J.D. Rodwell et al.,"Site-specific covalent modification of monoclonal antibodies: In vitro and in vivo evaluations", pages 2632-2636, see entire document.	1-28
Y	Annual Review of Biochemistry, Volume 60, Issued April 1991, Y.N. Vaishnau et al.," The biochemistry of AIDS",pages 577-630, see entire document.	21,22

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 SEPTEMBER 1993	07 SEP 1993

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. NOT APPLICABLE

Authorized officer
DAVID LACEY
Telephone No. (703) 308 0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Gene Analysis Techniques, Volume 5, issued May 1988, W.S. Thompson et al.," Antibodies introduced into living cells with liposomes localize specifically and inhibit specific intracellular processes", pages 73-79, see entire document.	1-28
Y	WO, A, 90/10448 (Bischofberger et al.) 20 September 1990, see entire document.	1-12, 25-27

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